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Distinct conformational stability and functional activity of four highly homologous endonuclease colicins

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Abstract

The family of conserved colicin DNases E2, E7, E8, and E9 are microbial toxins that kill bacteria through random degradation of the chromosomal DNA. In the present work, we compare side by side the conformational stabilities of these four highly homologous colicin DNases. Our results indicate that the apo-forms of these colicins are at room temperature and neutral pH in a dynamic conformational equilibrium between at least two quite distinct conformers. We show that the thermal stabilities of the apo-proteins differ by up to 20°C. The observed differences correlate with the observed conformational behavior, that is, the tendency of the protein to form either an open, less stable or closed, more stable conformation in solution, as deduced by both tryptophan accessibility studies and electrospray ionization mass spectrometry. Given these surprising structural differences, we next probed the catalytic activity of the four DNases and also observed a significant variation in relative activities. However, no unequivocal link between the activity of the protein and its thermal and structural stability could easily be made. The observed differences in conformational and functional properties of the four colicin DNases are surprising given that they are a closely related (≥65% identity) family of enzymes containing a highly conserved (ββα-Me) active site motif. The different behavior of the apo-enzymes must therefore most likely depend on more subtle changes in amino acid sequences, most likely in the exosite region (residues 72–98) that is required for specific high-affinity binding of the cognate immunity protein.

Keywords: colicins; endonucleases; protein folding; conformational stability; ESI-MS

Colicins are a group of toxins produced by *Escherichia coli* to kill other *E. coli* strains and related bacteria in order to gain a competitive advantage under nutrient stress conditions (James et al. 2002). Colicins comprise a three-domain organization with the role of two of the domains (the receptor binding and translocation domains) being to bind the target cell and allow the third domain (the cytotoxic domain) to translocate to its site of action, which can be either the periplasm, inner membrane, or cytoplasm depending on the colicin (James et al. 2002). A subgroup comprises colicins E2–E9, which act within the cytoplasm and kill the

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target cell through a nuclease activity. They are further subdivided into three sequence groups: (1) E2-like (comprising E2, E7, E8, and E9, within which their cytotoxic domains show strong sequence homology (≥65%)) having a Mg^{2+}-dependent, T-base-specific activity aimed at the chromosomal DNA, although with a weak, metal-ion-independent activity on RNA (Pommer et al. 2001; Walker et al. 2002); (2) E3-like (comprising E3, E4, and E6) cleaving the 16S rRNA at the ribosomal A site (between nucleotides 1493 and 1494; Bowman et al. 1971); and (3) E5, which cleaves a range of tRNA molecules (Ogawa et al. 1999).

The active DNase site of the E2, E7, E8, and E9 DNases comprises the last ~30 residues of the proteins (Fig. 1A), and has a fold made up of two antiparallel β-strands and an α-helix (Fig. 1B) and resembles a distorted zinc-finger. Consequently, the active site fold has been called the ββα-Me finger (Kuhlmann et al. 1999). This motif is found within nucleases spanning all biological kingdoms that otherwise have structurally diverse and unrelated protein scaffolds. Examples include apoptotic endonucleases (DNases), bacterial toxins, and homing endonucleases (Kuhlmann et al. 1999; Galburt and Stoddard 2002; Scholz et al. 2003). Biochemical analysis of colicin DNase function has previously focused on the E9 DNase (Pommer et al. 1998, 1999, 2001; Walker et al. 2002) and E7 DNase (Cheng et al. 2002).

The cytotoxic effect of colicins are prevented from killing their producing cell by the coexpression of an antidote called an immunity protein, which, in the case of the nuclease colicins, is a small (9–10 kD) globular protein that binds with high affinity to the cytotoxic domain (Wallis et al. 1995a; James et al. 1996; Walker et al. 2003). The competitive influence of the colicins is based on the specific tight binding of the cognate immunity protein over the different noncognate immunity proteins (Wallis et al. 1995a,b). In order to achieve this, a region of the DNase

A

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     1        10        20        30        40        50        60        70
E9   MESKRNKPGKATGKGKPVGDKWLDDAGKDSGAPIPDRIADKLRDKEFKSFDDFRKAVWEEVSKDPELSKNLNPSNKSSV
E8   MESKRNKPGKATGKGKPVGDKWLDDAGKDSGAPIPDRIADKLRDKEFKNFDDFRRKFWEEVSKDPELSKQFNPGNKKRL
E7   MESKRNKPGKATGKGKPVNNKWLNNAGKDLGSPVPDRIANKLRDKEFKSFDDFRKKFWEEVSKDPELSKQFSRNNNDRM
E2   MESKRNKPGKATGKGKPVGDKWLDDAGKDSGAPIPDRIADKLRDKEFKNFDDFRKKFWEEVSKDPDLSKQFKGSNKTNI
Cons MESKRNKPGKATGKGKPV..KWL..AGKD.G.P.PDRIA.KLRDKEFK.FDDFR...WEEVSKDP.LSK.....N....

     80        90        100       110       120       130
E9   SKGYSPFTPKNQQVGGRKVYELHHDKPISQGGEVYDMDNIRVTTPKRHIDIHRGK
E8   SQGLAPRARNKDTVGGRRSFELHHDKPISQDGGVYDMDNLRITTPKRHIDIHRGQ
E7   KVGKAPKTRTQDVSGKRTSFELHHEKPISQNGGVYDMDNISVVTPKRHIDIHRGK
E2   QKGKAPFARKKDQVGGRERFELHHDKPISQDGGVYDMNNIRVTTPKRHIDIHRGK
Cons ..G..P.......G.R...ELHH.KPISQ.G.VYDM.N....TPKRHIDIHRG.

                        ←——— Active Site ———→
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B

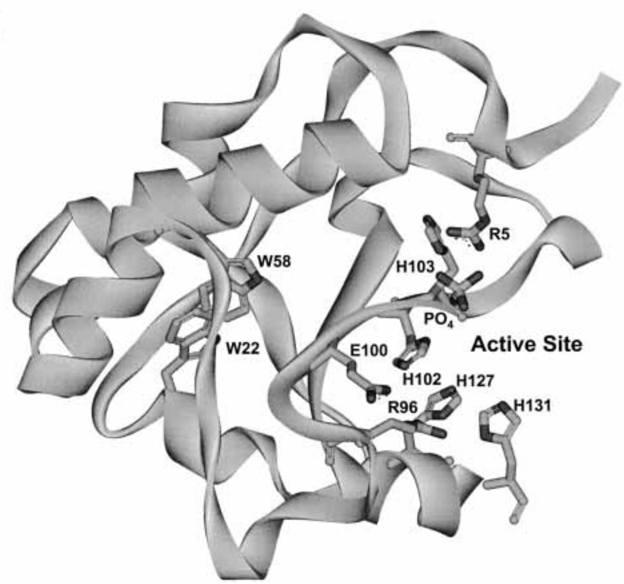

Figure 1. (*A*) Sequence alignments for the E colicin DNase domains E2, E7, E8, and E9. (*B*) The crystal structure of the apo-E9 DNase (Kuhlmann et al. 2000) showing the two buried tryptophans (W22 and W58) and the active site residues that are essential for the Mg^{2+}-dependent DNase activity (Walker et al. 2002).

domain (residues 72–98; Fig. 1A) has a variable sequence, although the rest of the protein is highly conserved, allowing specific binding interactions to be achieved.

Given that the E2 group is the most numerous E colicin nuclease group and is the best characterized, both structurally, with crystal structures of E7 and E9 available (Kleanthous et al. 1999; Ko et al. 1999), and functionally (Pommer et al. 1998, 1999, 2001; Cheng et al. 2002; Walker et al. 2002), we focused on this group in the present work. Specifically, we investigated structural features of the four isolated related 15-kD cytotoxic DNase domains by assessing their thermal stabilities and conformational properties in solution by differential scanning calorimetry (DSC), tryptophan accessibility studies, and electrospray ionization mass spectrometry (ESI-MS). Given the remarkable outcome, which revealed strikingly different thermal and conformational stabilities of the four apo-DNases, we also compared their enzymatic activities and observed a significant variation. Therefore, the present studies supplement colicin structural and functional biology because, in contrast to E7 and E9, comparatively little biophysical and biochemical characterization has been carried out for E2 and E8.

More generally, the present study reiterates that conformational dynamics, thermal stabilities, and functional activities of proteins are impossible to predict purely from primary sequence information. In particular, in this case, the four colicins have a high degree of sequence conservation within the active site, with all of the residues critical for catalysis being absolutely conserved (Walker et al. 2002). The observed differences in stability and activity therefore most likely originate from differences in the variable region, which is involved in cognate immunity protein recognition. A better understanding of the physicochemical characteristics of the cytotoxic domain of these enzymes is important, as the structural flexibility has earlier been proposed to be of importance in the process of translocation to its site of action (Pommer et al. 1999).

Results

Conformational variability monitored by tryptophan fluorescence quenching

To investigate and characterize structural features of the DNases in solution, we used two independent techniques, tryptophan fluorescence quenching (this section) and (nano) ESI-MS (following section) to address the conformation of the proteins. Colicin DNases contain two strictly conserved tryptophans that are buried within the interior of the protein. Consequently, as typical for tryptophans buried in the interior of folded proteins, the fluorescence emission wavelength maxima for the four DNases are in the range 333–336 nm. In such a case, their accessibility to the noncharged collisional quencher acrylamide should be dependent on the global conformational state of the protein, being high for more open conformational states but low for closed conformers. N-Acetyl-L-tryptophanamide (NATA) was used as a reference compound that is fully and easily accessible to the quencher and is characterized by a high Stern-Volmer quenching constant (K_{SV}). Results for the steady-state acrylamide quenching of apo- and holo- E2, E7, E8, and E9 are summarized in Figure 2 and Table 1, along with the NATA control. To further extend this analysis, we determined k_q, the bimolecular collisional rate constant, which essentially represents the average number of collisional encounters between the tryptophans and acrylamide and, thus, is a quantification of their accessibilities. In order to do this, we determined the average lifetime fluorescence $<\tau>$ for each wild-type DNase in the absence of quencher by using time-resolved fluorescence because k_q equals $K_{SV} / <\tau>$. The recovered parameters for intensity decay and the collisional quenching constants are shown in Table 2 with each apo-DNase exhibiting a heterogeneous decay, described by a sum of three exponential terms ($p_1,p_2,p_3 / \tau_1,\tau_2,\tau_3$). The four apo-DNases show marked differences in their sensitivity to acrylamide quenching, implying that their structures have different relative degrees of compactness. On the basis of the present assay, apo-E7 has a significantly more open structure than the other apo-DNases because the tryptophans are more accessible to the quencher (k_q is 1.8-fold greater than for E9 and ~3.5-fold greater for E2 and E8). Summarizing, the combination of fluorescence quenching and lifetime measurements suggest that the conformational compactness of the four colicins is strikingly different and follow the order E7 < E9 < E2 ≈ E8.

On Zn^{2+} binding, all of the colicin DNases show a significant decrease in K_{SV} values, to a more or less uniform value (Table 1), indicating that metal binding results in substantial protection of the tryptophans. Thus, the fluores-

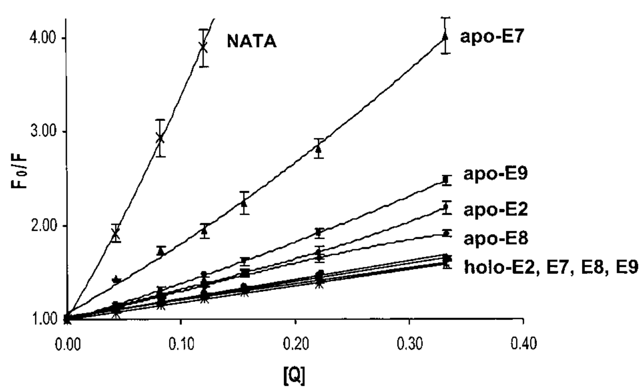

Figure 2. Stern-Volmer plots of the quenching of the fluorescence of NATA and DNases E2, E7, E8, and E9 in the presence (holo) and absence (apo) of Zn^{2+} ions by acrylamide. The data were fitted by nonlinear regression of three sets of measurements using a modification of the Stern-Volmer equation: $F_0 / F = 1 + K_{sv}[Q]e^{V \cdot [Q]}$.

Table 1. *Acrylamide quenching parameters for E colicin DNases*

	apo DNases			holo DNases		
Protein	K_{SV} (M^{-1})	V (M^{-1})	r^2	K_{SV} (M^{-1})	V (M^{-1})	r^2
E2	2.79 ± 0.15	0.74 ± 0.19	0.993	2.41 ± 0.11	[a]	0.993
E7	7.37 ± 0.48	0.62 ± 0.24	0.989	2.45 ± 0.09	[a]	0.993
E8	3.13 ± 0.11	[a]	0.995	1.84 ± 0.08	[a]	0.994
E9	3.49 ± 0.13	0.75 ± 0.14	0.996	2.29 ± 0.11	[a]	0.991
NATA	20.55 ± 0.91	1.34 ± 0.15	0.994			

Data were fitted according to $F_0/F = 1 + K_{SV} \cdot [Q] \cdot e^{V[Q]}$.
[a] Static quenching component not observed.
(NATA) N-acetyl-L-tryptophanamide.

cence quenching measurements suggest that all four holoenzymes share a similar conformational compactness. We therefore measured only the lifetime of the Zn^{2+}-containing E9 DNase, which revealed an average lifetime of 3.7 nsec (data not shown) and a k_q value of 0.62×10^9 $M^{-1}sec^{-1}$. This latter value indicates an ~25% decrease in tryptophan accessibility when compared with apo-E9 DNase. This change in accessibility for colicin E9 correlates well with the increased thermal stability induced by metal ion binding (Pommer et al. 1999).

Conformational variability monitored by (nano) ESI-MS

In the previous section, we related the accessibilities of the buried tryptophans to the global structure of the proteins. However, because local, in addition to global, structural features may affect the accessibility of the tryptophans, we next used ESI-MS to address the global structural conformation of the DNases. This technique provides some unique features for the investigation of conformational properties of proteins, as it has the ability to simultaneously provide information about the conformation of the protein and ligand binding (Veenstra 1999; Kaltashov and Eyles 2002; Konermann and Simmons 2003). Additionally, it has an advantage over other techniques, such as circular dichroism and steady-state fluorimetry, in that subpopulations of protein conformers can be analyzed simultaneously instead of as the ensemble of different subpopulations. The relevance to the present study is that, under certain conditions, ESI-MS can reveal the dynamic features of conformational stability. Importantly, despite being a gas-phase-based technique, results from ESI-MS experiments can, when carefully evaluated, be informative about solution phase properties (Loo 1997, 2001; Hernandez and Robinson 2001).

We have previously used ESI-MS to investigate the E9 DNase (van den Bremer et al. 2002). In the present work, we compare these previous results with those obtained for the E2, E7, and E8 DNases (Fig. 3). As before, samples are

Table 2. *Fluorescence lifetime data analysis*

apo protein	$p_1p_2p_3$[a]	$\tau_1\tau_2\tau_3$ (nsec)[b]	χ^2	$\langle\tau\rangle$ (nsec)	k_q (10^9 M^{-1} sec^{-1})[c]
E2	0.069	0.91 ± 0.16	1.069	6.63 ± 0.09	0.42 ± 0.17
	0.160	3.52 ± 0.46			
	0.771	7.78 ± 0.06			
E7	0.131	0.87 ± 0.06	1.075	4.87 ± 0.06	1.51 ± 0.48
	0.309	3.60 ± 0.18			
	0.560	6.50 ± 0.05			
E8	0.059	0.53 ± 0.10	1.070	6.96 ± 0.07	0.45 ± 0.13
	0.102	3.58 ± 0.49			
	0.839	7.82 ± 0.05			
E9	0.159	0.78 ± 0.05	1.060	4.21 ± 0.03	0.83 ± 0.13
	0.397	2.91 ± 0.07			
	0.450	6.53 ± 0.03			

[a] Normalized *P*-values representing fractional contributions.
[b] The excited-state lifetimes, $\tau_1\tau_2\tau_3$, were determined from single-photon timing measurements (data not shown).
[c] Biomolecular rate constant for acrylamide quenching was calculated using $\langle\tau\rangle$ and K_{SV} (see Table 1).
The χ^2 values are "reduced chi-squares," indicating goodness of fit.

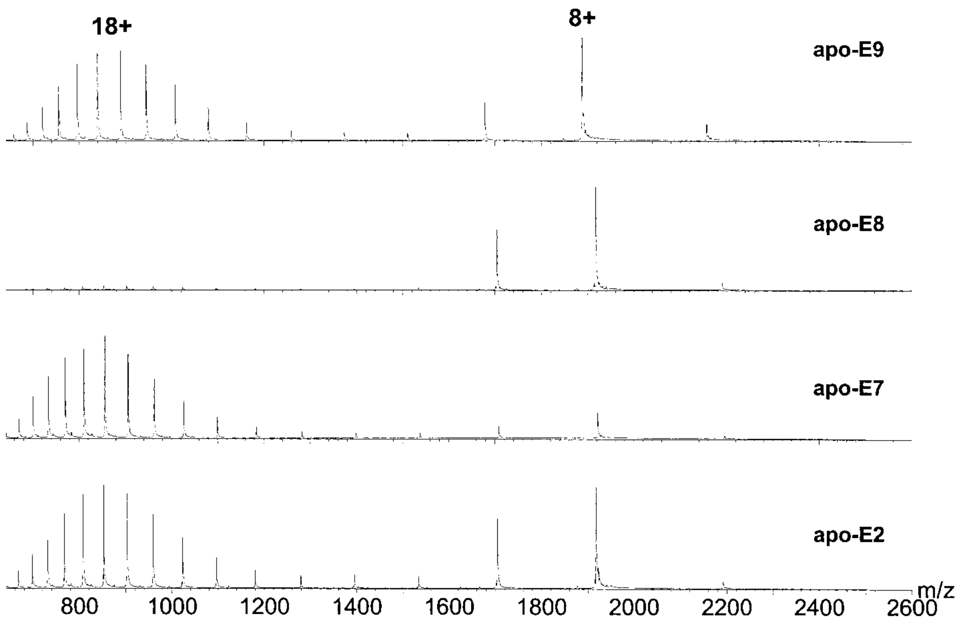

Figure 3. Nano-electrospray ionization (ESI) mass spectra of the apo-DNases E2, E7, E8, and E9 (10 μM) sprayed from an aqueous 50-mM ammonium acetate solution (pH 7.4).

being electrosprayed from solutions under conditions that are known to preserve "native" conformations (see also Materials and Methods). In the ESI-MS process, proteins become ionized through multiple protonations (Fenn et al. 1989; Smith et al. 1990) with the resulting mass spectrum typically displaying a single continuous ("Gaussian") charge state envelope. However, for all of the colicin DNases, a bimodal charge distribution is observed comprising a broad distribution encompassing ion peaks from 10+ to 23+ (with a maximum located at 18+), and a second narrow distribution of three ion peaks (7+, 8+, 9+) with a maximum located at 8+ (Fig. 3). In each case, both of the charge distributions produce a calculated mass for the protein identical to the theoretical mass based on the amino acid sequence. However, the relative abundance of each distribution is different for the four colicin DNases, with the high charge state distribution (around 18+) being dominant for the E7 DNase, and the low charge state (around 8+) being dominant for E8. The charge distributions observed for the DNases E2 and E9 are found to be somewhat intermediate. Several control experiments were carried out to exclude the possibility that the differences observed in the mass spectra were the result of unforeseen experimental artefacts such as variations in spray conditions. The results of one of those control experiments is given in Figure 4, where the mass spectrum of an equimolar mixture of the apo-E7 and E8 DNases is shown. The spectrum resembles a combination of the two spectra obtained for the isolated DNases (Fig. 3), confirming the strikingly different behavior of apo-E7 and apo-E8 in ESI-MS.

The extent of protonation of a protein during the ESI process is dependent on the number of basic sites at the surface of the proteins and thus also on the conformation/structure of the protein (Konermann and Simmons 2003). In general, proteins that exhibit unfolded, open, or flexible structures become more protonated than proteins with compact, highly folded structures (Chowdhury et al. 1990; Przybylski and Glocker 1996; van den Bremer et al. 2002). The observed 18 charges on a 15-kD protein is atypical and very high for a protein sprayed from buffered neutral ammonium acetate solutions (Heck and Van den Heuvel 2004), which must indicate that the protein is in a solution-phase conformation that is likely very open. In our original ESI-MS study on the E9 DNase (van den Bremer et al. 2002), we assigned the low charge distribution (states 7+, 8+, and 9+) as originating from compact, folded-like, conformational states (termed F). We assigned the high charge distribution (11+ to 22+) as originating from a population of open, more unfolded, conformational states (termed U). We showed by hydrogen-deuterium exchange mass spectrometry that these two conformers are in rapid equilibrium (on the seconds timescale; van den Bremer et al. 2002). A semiquantitative assessment of the relative contribution of the two distinct populations of conformers may be given by the ratio of their summed peak areas [A_F / (A_U + A_F); Mirza et al. 1993; van den Bremer et al. 2002]. Such an approach provides values of 0.05, 0.29, 0.32, and 0.92 for the E7, E9, E2, and E8 DNases, respectively, indicating that E7 is, on average, predominantly in its highly unfolded conformational state, whereas E8 is in time on average more in its compact con-

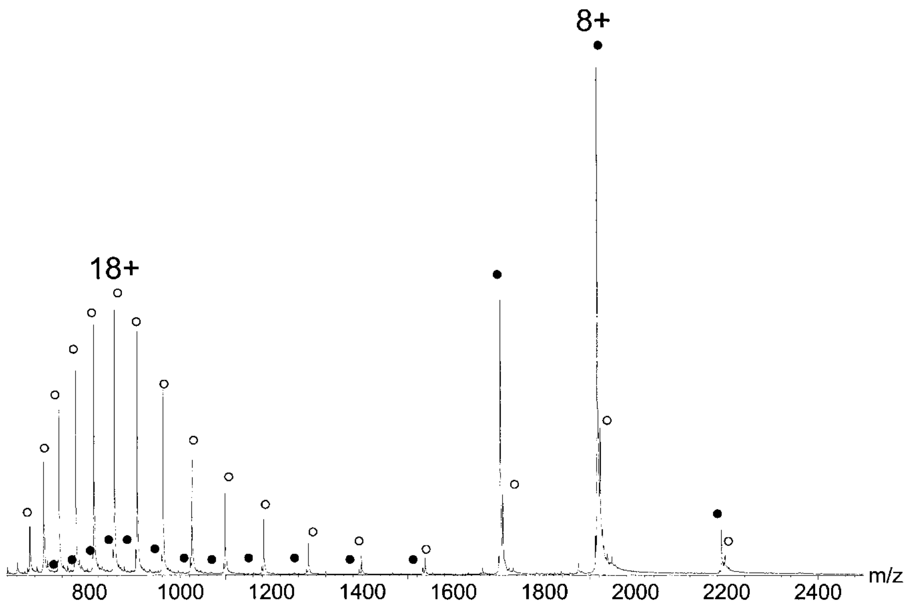

Figure 4. Nano-electrospray ionization (ESI) mass spectra of an equimolar mixture (10 μM each) of apo-DNases E7 and E8 sprayed from an aqueous 50 mM ammonium acetate solution (pH 7.4). Ion peaks representing DNases apo-E7 and E8 are highlighted by open and filled circles, respectively.

formational state. Summarizing, the conformational equilibrium observed follows, when ranked from mostly unfolded to more folded, the order E7 < E9 ≈ E2 < E8.

Some care must be taken when relating the relative sizes of the two populations in the mass spectra to the fractional contributions of the protein conformers in solution because unfolded/open-like states are usually detected more readily (Cech and Enke 2000, 2001; Dobo and Kaltashov 2001; van den Bremer et al. 2002). Nevertheless, the values for the relative fractions of folded conformers correlate very well with the tryptophan accessibility experiments of the apo-DNases. Both tryptophan fluorescence quenching and mass spectrometric experiments indicate that the global structures show different relative levels of compactness. The ESI-MS results imply that the E2 DNase is more unfolded compared with the E8 Dnase, in contrast to the similarity in the degrees of solvent protection of the tryptophans (Table 1). However, E8 experiences only one type of fluorescence quenching, whereas E2 experiences two types (i.e., dynamic and static), suggesting that the E2 structure is more open than E8, and thus follows the same order of our ESI-MS findings.

The E9 DNase, which has an nM affinity for Zn^{2+}, undergoes metal ion induced conformational changes that are localized near the active site of the protein (Hannan et al. 2000; Kuhlmann et al. 2000; Keeble et al. 2002) and result in increased stability (Pommer et al. 1999). This is reflected in the ESI-MS spectra, which show, besides the observed mass increase of 63 D (65 D minus $2H^+$) due to specific binding of one Zn^{2+} ion, a change in the charge distributions in the holo-E9 DNase, so that only the low-charge distribution was observed (van den Bremer et al. 2002). In the present work, we sprayed the other colicin DNases in the presence of a fivefold excess of Zn^{2+} and also observed that only the low-charge distribution is detected (data not shown). These results are also in agreement with the tryptophan fluorescence quenching results obtained for the holo-enzymes, as described in the previous section. In contrast, when experiments were repeated with the apo-DNases sprayed in the presence of a large excess of Mg^{2+}, the natural cofactor, the ESI-MS spectra were identical to those of the apo-DNases alone. Thus, Mg^{2+} does not significantly bind nor does it stabilize the apo-DNases. This absence of binding is in agreement with previous isothermal titration calorimetry experiments in which no Mg^{2+} binding could be observed, indicating that binding was very much weaker than mM (Pommer et al. 1999). Therefore, specific Mg^{2+} binding to E9 DNase in vivo is unlikely.

Thermal stability probed by differential scanning calorimetry

Given the observed differences in the conformational properties of the four colicin DNases, we addressed their thermodynamic stabilities and therefore investigated the thermal unfolding of the colicin DNases in both the apo-(metal free) and holo-forms by DSC. The results are summarized in Table 3. The midpoint melting temperatures (T_m) for the apo- (36.6°C) and transition metal bound-E9 DNase (63°C) determined in the present work are in good agreement with

Table 3. *Calorimetric data of colicin DNases in the presence and absence of Zn^{2+}*

DNase	apo T_m (°C)	holo T_m (°C)
E2	37.3	61.2
E7	26.3	59.9
E8	45.5	63.7
E9	36.6	63.0

the values of Pommer et al. (1999). In all cases, the holo-DNases were found to be much more stable than the apo-DNases, and to have similar T_m values (60°C–64°C). In contrast, the T_m values vary quite considerably between the apo-DNases, 26.3°C for apo-E7 DNase to 45.5°C for the apo-E8 DNase, with the T_m of E2 DNase being similar to that of E9 DNase (37°C). Hence, despite their high sequence identities, the apo-DNases have significantly different thermal stabilities, which may be of biological importance, as they are observed to vary to both sides of the biologically important mammal body temperature of 37°C. It is interesting to note that the observed order of T_m values for the apo-DNases is E7 < E9 ≈ E2 < E8, very similar to the extent of unfolding/folding observed in the electrospray mass spectra.

Comparison of the enzymatic activities of the colicin DNases

A priori we supposed that these four proteins have identical folding properties and thus similar enzymatic activities. Now that we observed these striking differences, we decided to probe the DNase activities also as a function of temperature. We have previously used two different assays to monitor DNA cleavage, each using a different type of DNA substrate: the spectrophotometric Kunitz assay to monitor double-strand cleavage of linear calf thymus dsDNA, and a nicking assay using supercoiled pUC18 to monitor single-strand cleavage. In the present work, we have used the assays (Fig. 5) as a semiquantitative comparison of the relative catalytic activities, an approach we have previously used when comparing active site mutants of the E9 DNase (Walker et al. 2002). We carried out the assays on the four apo-colicin DNases in the presence of Mg^{2+}, the physiologically relevant divalent metal ion for DNA cleavage (Walker et al. 2002). As Mg^{2+} does not significantly bind, these assays provide data on the activity of the non-metal bound DNases. The assays were carried out over a range of temperatures from 20°C to 70°C. In the Kunitz assay the E2, E7, and E9 DNases show similar levels of activity, although the E2 and E9 DNases have a higher optimal temperature (~50°C) than the E7 DNase (~40°C). In contrast, the E8 DNase shows both a higher activity (up to 10-fold) at all temperatures tested, and a higher optimal temperature (~60°C). The temperature dependences of the activities from the plasmid-nicking assay essentially parallel these results. However, the DNases show different nicking profiles, with E8 DNase showing activity over the whole temperature range and being most active at 60°C, where it ran out of supercoiled plasmid DNA substrate. DNases E2 and E9 have their optimum at 40°C. At this temperature, both DNases produce mostly linear products; however, E9 DNase ran out of supercoiled plasmid DNA substrate, whereas the E2 DNase did not. This indicates that at 40°C the E9 DNase is more active compared with E2. Linear cleavage products were not observed for E7 at any assayed temperatures, indicating a weaker activity. Summarizing, the catalytic properties and activities of the four apo-colicin DNases differ strikingly, not only in catalytic rates, but also in selectivity and temperature dependence. In contrast, no activity at any temperature was observed for any of the DNases in the presence of Zn^{2+} (data not shown). Although this metal ion binds with a high affinity to the active site,

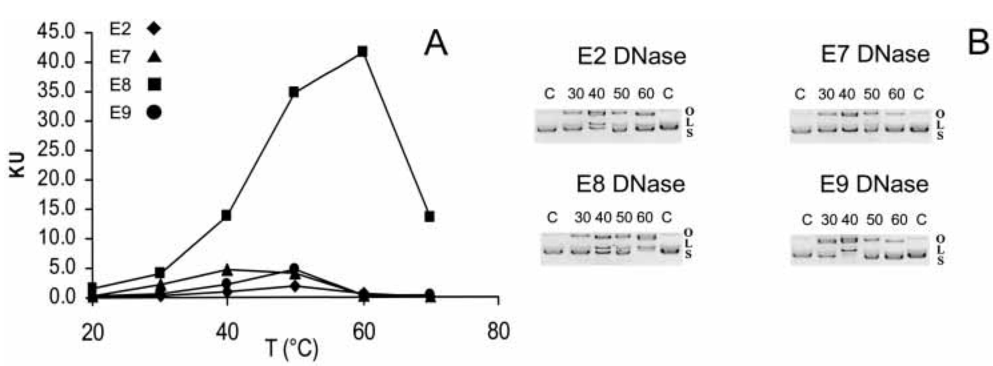

Figure 5. (*A*) Temperature-dependent Kunitz activity assays in the presence of 20 mM Mg^{2+} in 50 mM triethanolamine buffer (pH 7.4; 1 KU = 0.001 $\Delta A_{260} min^{-1} \mu g^{-1}$ protein). (*B*) Plasmid-nicking assays: Each panel indicates the migration of supercoiled (S), open circular, and linear DNA (L) after 10 min incubation. Control (C) is an identical reaction in the absence of Mg^{2+} ions.

this observed inactivity is in line with our previous studies on the E9 DNase (Pommer et al. 1999).

Discussion

In the present work we have probed the sequence-structure-function relationships within a group of highly similar proteins—the colicin DNases. The advantage of analyzing a group of proteins is that the conserved features of structure and function can be ascertained, as well as the differences. Despite a high degree of sequence identity (≥65%) and structural homology, we find that both the structural and functional properties of the proteins vary widely. The observed thermal stabilities of the colicin apo-proteins vary in a manner consistent with their conformational state, as observed by fluorescence quenching and electrospray mass spectrometry. These differences were unexpected, although differences have also been seen previously for mammalian apo-myoglobins, whereas the holo-variants are structurally and functionally similar (Scott et al. 2000).

Some members of the E colicin nuclease group have been quite well characterized by X-ray crystallography with crystal structures of E7 and E9 available (Kleanthous et al. 1999; Ko et al. 1999). These structures are highly similar and show no striking differences. Therefore, the observed differences in structural and functional behavior described in the present work cannot be explained on the basis of these X-ray structures. We believe this is caused by the fact that in solution there is a dynamic conformational equilibrium, whereas in X-ray crystallography primarily only a static lowest energy structure is probed.

Given the fact that the other parts of the sequences are highly similar, the differences in behavior are most likely a result of the sequence variation within the exosite (residues 72–98) that is required for specific high affinity binding of the cognate immunity protein. The greater sequence variation in this area is a consequence of the evolution of highly specific binding of the cognate immunity protein over other, structurally similar, noncognate immunity proteins. The hypothesis that this part of the sequence may be important in explaining the observed variation is supported by findings in the co-crystal structure of the E9 DNase with single-stranded DNA, which shows that residue Tyr 83, which is nonconserved and a putative specificity-determining residue for binding immunity proteins (Curtis and James 1991), intercalates with the DNA (Kolade et al. 2002). The precise molecular origins of how the catalytic activities and thermal stabilities/conformational states are produced is beyond the scope of the present study but are the focus of ongoing investigations.

A remaining question is whether the observed differences in conformational stability and activity of the apo-DNase domains of the four wild-type colicins are correlated and whether they have biological implications. It has been shown that the ability to insert and pass through the inner membrane (as judged by an in vitro lipid bilayer experiment) is similar for all colicins, both in the apo- and holo-form (Mosbahi et al. 2002), and thus likely independent of thermal and structural stability. This argues against the proposal by Pommer et al. (1999) that a structurally destabilized DNase domain might be important for colicin DNase uptake into the target cell. Additionally, all colicin DNases have temperature optima at or above 40°C (Fig. 5), which fits with the expected temperature of their natural in vivo environment (37°C–40°C). The conformationally most stable colicin DNase E8 shows by far the highest in vitro activity in the present Kunitz assay (Fig. 5). However, the impact of this on the in vivo cytotoxicity is unknown and is subject for further analysis.

In summary, we have demonstrated that there are marked differences in the conformational properties and activities of the four apo-colicin DNases. These differences were a priori unexpected, given (1) the high sequence identity of the colicin DNases in the active site region (Fig. 1), whereby all of the residues identified as being essential for activity (Walker et al. 2002) are absolutely conserved and (2) the high similarities between the known X-ray crystal structures of the DNase domains. Therefore, an important observation of this study is that the correlation between (highly similar) sequence information on the one hand and dynamic structural features and activity on the other hand is not self-evident.

Materials and methods

Colicin DNase domains E2, E7, E8, and E9 were expressed in *E. coli* and purified as previously described (Walker et al. 2002). Confirmation of each expressed DNase (E2, E7, E8, and E9) by nano ESI-MS under denaturing conditions (50% (v/v) acetonitrile containing 0.1% formic acid) yielded average masses of 15,329.9 ± 0.4 D, 15,374.7 ± 0.3 D, 15,322.6 ± 0.4 D, and 15,088.2 ± 0.3 D, respectively. These masses are all within 1 D of those calculated from the amino acid sequence.

Nano electrospray ionization mass spectrometry

Time-of-flight electrospray ionization mass spectra were recorded on a Micromass LC-T mass spectrometer operating in the positive ion mode. Prior to analysis, a 600–3000 m/z scale was calibrated with CsI (2 mg/mL) in isopropanol/water (1:1). Samples for charge state distribution analysis were introduced via a nanoflow electrospray source. Nanoelectrospray needles were prepared as described previously (van den Bremer et al. 2002). Unless stated otherwise, all samples were dissolved in 50 mM aqueous ammonium acetate solutions at pH 7.4. In all experiments, an aliquot (1–3 μL) of protein sample at a concentration of 10 μM was introduced into the electrospray needles. The nanospray needle potential was typically set to 1200 V and the cone voltage to 30 V. The mass spectrometer was operated without source heating. During individual titration experiments, all parameters of the mass spectrometer were kept constant (van den Bremer et al. 2002).

Steady-state fluorescence spectroscopy

Quenching of tryptophan fluorescence by acrylamide in the range 0–0.33 M was performed by adding aliquots of acrylamide (1.4 M in 50 mM ammonium acetate) to a solution containing 6 μM DNase. For experiments on the holo-proteins, 46 μM of zinc acetate was added. To avoid interference by acrylamide absorption, we set the excitation wavelength at 300 nm. The fluorescence intensity was monitored at 345 nm. Measurements were performed with an LS-50 Luminescence spectrophotometer (Perkin Elmer) at 20°C. In all studies, excitation and emission bandwidths were set at 5.0 and 7.0 nm, respectively. Spectra were corrected for dilution and background. The data were analyzed by a modified form of the Stern-Volmer equation:

$$F_0 /F = 1 + K_{SV} [Q] e^{V[Q]},$$

where F_0 and F are the fluorescence intensities in the absence and presence, respectively, of quencher (acrylamide) at concentration [Q], K_{SV} is the Stern-Volmer constant for dynamic quenching, and V is a constant representing static contributions to the quenching. In addition, K_{SV} is equal to $k_q\langle\tau\rangle$, where k_q is the apparent bimolecular rate constant for the collision of the quencher and the protein, and $\langle\tau\rangle$ is the average excited-state lifetime of the tryptophan residues in the absence of quencher. We used the average lifetime $\langle\tau\rangle$ taken at 349 nm emission wavelength to calculate k_q (Lakowicz 1999).

Time-resolved fluorescence spectroscopy

Time-resolved fluorescence decay times were measured in a home-built setup with mode-locked continuous wave laser excitation and time-correlated photon counting detection. The pump laser was a CW diode-pumped, frequency-doubled Nd:YVO_4. The mode-locked laser was a titanium: sapphire laser coupled with a pulse picker that decreased the repetition rate of the excitation pulses to 3.8×10^6 pulses per second. The maximum pulse energy was a few pJ, the wavelength 295 nm, and the pulse duration 3 psec. The temperature was controlled and set on 20°C. Fused silica cuvettes of 10-mm light path were used. The fluorescence emission was collected at 348.8 nm at an angle of 90° with respect to the direction of the excitation light beam.

Experimental data consisted of repeating sequences of measurements of the polarized emission (parallel and perpendicular component) fluorescence decays of the reference compound (three cycles of 20 sec), the protein sample (10 cycles of 20 sec), the background (two cycles of 20 sec), and again the reference compound. In that way, an eventual temporal shift can be traced and corrected. All cuvettes were carefully cleaned and checked for background luminescence prior to the measurements. For obtaining a dynamic instrumental response of the setup, the single exponential fluorescence decay was measured of paraterphenyl in a mixture of cyclohexane and CCl_4 in a 50/50% volume ratio. For further details, see Visser et al. (1994). Data analysis was performed using a home-built computer program (Digris et al. 1999; Novikov et al. 1999).

Differential scanning calorimetry

DSC was used to measure transition temperatures (T_m) of the DNases in the presence and absence of Zn^{2+}. T_m is defined as the temperature at which the excess heat capacity is maximal. Lyophilized proteins were diluted in 50 mM ammonium acetate (pH 7.4). Excess heat (C_p) versus temperature scans were obtained from 0.3 mg/mL (~20 μM) protein solutions using a high-sensitivity differential scanning calorimeter MicroCal VP-DSC (MicroCal, Inc.). The sample and reference solutions were carefully degassed under vacuum for 15 min before loading the cells (0.514 mL). Prior to each analysis, the system was equilibrated for 20 min at 15°C. During measurements, the temperature was increased from 15°C to 80°C at scan rates of 1°C/min.

Kunitz assay

Calf thymus DNA (~50 μg DNA mL^{-1} to give a final A_{260} of 1) was made up in 50 mM triethanolamine buffer (pH 7.4) containing 10 mM $MgCl_2$ to assay the DNase activity. Ten micrograms of DNase was used per 1.0 mL for each assay. Prior to the analysis of the enzymatic reaction, the enzyme was preincubated at the temperature of interest. Reactions were initiated by addition of the enzyme and the ΔA_{260} was observed over 600 sec in a dual beam UV/Visible Cintra10 spectrophotometer (GBC Scientific Equipment Pty Ltd.) that was thermostated at different temperatures by using a Peltier-element. The reference cuvette contained identical amounts of calf thymus DNA and metal ion as the sample cuvette. Data were downloaded and processed with Microsoft Excel. Activities were calculated as $\Delta A_{260} min^{-1} \mu g^{-1}$ protein and converted to Kunitz units (KU), where 1 KU = 0.001 $\Delta A_{260} min^{-1} \mu g^{-1}$ protein.

Plasmid nicking assay

Assays were performed in 50 mM triethanolamine buffer (pH 7.4) containing ~1 μg of plasmid DNA (pUC18) and 20 mM $MgCl_2$. Reactions were started by the addition of E2, E7, E8, or E9 DNase and incubated for 10 min at different temperatures. The reactions were stopped by adding 5 μL stop mix (containing EDTA) before electrophoresis in a

1.2% (w/v) agarose gel. Gels were stained with ethidium bromide.

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